7 Metabolism of nitrogen-containing compounds

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The way in which the rumen has evolved as their first digestive organ potentially affords ruminants an efficiency of protein nutrition that is not available to non-ruminant herbivores. Protein is synthesized in the gut in the form of rumen microorganisms. The necessary energy is derived from plant polysaccharides such as cellulose, and the nitrogen is derived from ammonia and amino acids in the rumen. The energy and nitrogen sources can therefore be substrates of little value to most non-ruminants. Even more important, however, is the direct availability of that microbial protein for digestion and absorption by the host animal. Herbivores which employ hind-gut fermentation can only achieve the same efficiency of microbial protein utilization by coprophagy. In contrast, microbial protein is generally the ruminant's principal source of amino acids.

The benefit actually derived from the rumen fermentation, in terms of protein nutrition, varies according to the diet. Virtanen (1966) elegantly demonstrated that cows can maintain unimpaired milk production on diets lacking protein and with cellulose as principal carbon source and urea as the main nitrogenous nutrient. Yet, especially with intensive production systems, the nitrogen metabolism of rumen microorganisms is usually regarded as being inefficient. Dietary protein is broken down much too rapidly relative to the breakdown of the energy-containing plant fibre, excessive ammonia production results, and the biological value of the dietary protein is severely reduced.

The objectives of research in this field have therefore been twofold. One is to capitalize on the microbial capacity to form protein from ammonia by feeding non-protein nitrogen (NPN). The other is to minimize protein

breakdown in the rumen, and thereby to increase the 'escape' dietary protein (i.e. protein unaffected by passage through the rumen) reaching the lower tract.

7.1 Flow of nitrogenous compounds through the rumen

It is customary for ruminant nutritionists to determine the nitrogen content of feeds by a Kjeldahl procedure, and then to multiply that value by 6.25 and call it the 'crude protein' content of the diet (Ørskov, 1982). This is a useful device for evaluating diets, but clearly oversimplifies the very complex nitrogenous components of plant materials. Titles such as 'Urea as a Protein Supplement' (Briggs, 1967) might well puzzle the uninitiated.

The plant materials that comprise the bulk of ruminant feeds are composed of a vast array of nitrogenous compounds. Protein is almost always the most abundant, but nucleic acids will usually occur in association with protein, and substantial amounts of nitrate and ammonia may be present, depending on the diet. Plant materials may be supplemented with NPN in the form of compounds such as urea.

Other nitrogenous inputs to the rumen fermentation are salivary mucoprotein and urea; the latter enters both in saliva and by diffusion through the rumen wall. Gaseous nitrogen also enters the rumen, but nitrogen fixation is almost certainly not of quantitative significance.

The interconversions that occur in the rumen are illustrated for an example of sheep receiving a lucerne-chaff diet (Figure 7.1). This diagram ignores the distinction between crude protein and true protein or other

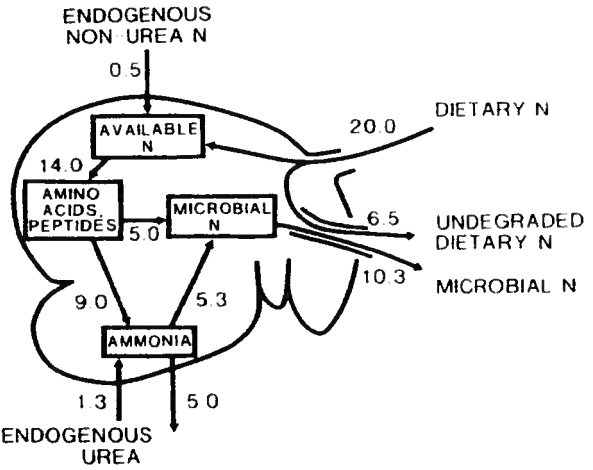

Figure 7.1 Flow of nitrogen (g day^{-1}) in the rumen of sheep receiving a lucerne-chaff diet (Nolan, 1975).

materials, and markedly different diets, e.g. straws or cereals, might give substantially different patterns, but the following general observations apply to most situations.

- All interconversions are generally considered to occur by the action of microbial enzymes.
- Microbial protein is the most abundant form of protein nitrogen leaving the rumen.
- Endogenous urea will only be of quantitative significance for microbial protein synthesis when dietary protein is low.
- Ammonia is the major product of catabolism, and also the main substrate for microbial protein synthesis.
- Ammonia overflow leads to inefficient nitrogen retention.

7.2 Breakdown of dietary nitrogenous compounds

7.2.1 Protein degradation

Dietary factors. Protein is the most abundant source of nitrogen in most ruminant diets. Feed protein is usually hydrolysed rapidly in the rumen, although the precise rate and extent of breakdown depend on a number of factors, which ultimately determine the nutritive value of the protein. Early work produced the general rule that protein breakdown in the rumen is proportional to solubility (Chalmers and Synge, 1954; Henderickx and Martin, 1963; Henderickx, 1976), but subsequent research has shown that other properties are also important (Kaufmann and Lupping, 1982). For example, some (pure) soluble proteins are broken down more slowly than insoluble proteins, depending on the degree of secondary and tertiary structure, and cleavage of disulphide bonds enhances the breakdown of albumin and similarly heavily cross-linked molecules (Mahadevan *et al.*, 1980; Nugent *et al.*, 1983; Wallace and Kopecny, 1983). Conversely, the introduction of artificial cross-links into proteins inhibits their hydrolysis (Friedman and Broderick, 1977; Wallace, 1983). Heating and formaldehyde treatments, affecting both solubility and cross-linking, have been used to protect proteins from rumen degradation and thereby provide bypass protein to the lower tract (Kaufmann and Lupping, 1982). However, these treatments may impair the subsequent availability of some amino acids, notably lysine, cysteine and tyrosine (Ashes *et al.*, 1984). Gentler methods, such as using tannin, or coating protein supplements with an undegradable shell of heated, dried blood, may be preferable (Ørskov *et al.*, 1980). The proteins in some plants often have some degree of natural protection, depending on the matrix in which they are located. The hydrolysis of non-protein polymers, such as polysaccharides, sometimes limits the access of proteolytic organisms to their substrate (Ganev *et al.*, 1979; Siddons and Paradine, 1981).

The nature of the diet has a major influence on the proteolytic activity of rumen contents. Fresh herbage promotes an activity up to nine times higher than that found with dry rations, the higher soluble-protein content of the herbage enriching for proteolytic bacteria (Nugent and Mangan, 1981; Hazlewood *et al.*, 1983; Nugent *et al.*, 1983). It has been suggested that endogenous plant proteases may play a significant role in the breakdown of fresh herbage protein in the rumen (Theodorou, 1995), largely on the grounds that such enzymes exist and are predominantly responsible for protein breakdown in the silo. Evidence of its significance relative to microbial activities in the rumen is not yet forthcoming. Cereal diets also yield higher proteolytic activities than do dry forage diets, probably because proteolytic rumen microorganisms tend to be amylolytic rather than cellulolytic (Siddons and Paradine, 1981). The nature of the protein substrate is another factor which affects proteolytic activity. Hydrolysis of leaf fraction 1 protein was stimulated, relative to casein, by fresh fodder, in which it would be abundant, more than by a diet consisting of hay plus concentrates (Hazlewood *et al.*, 1983; Nugent *et al.*, 1983). In contrast, when albumin replaced casein as an experimental protein supplement to a sheep diet, the rate of breakdown of albumin relative to casein was unchanged, despite a modified proteolytic flora (Wallace *et al.*, 1987*a*). Furthermore, the proteolytic activity of the rumen contents was hardly changed. The effect of different dietary proteins on rumen proteolysis probably varies from protein to protein and with the other constituents of the diet.

Cilate protozoa. The rumen ciliates have for many years been known to be proteolytic. Holotrichs undergo rapid endogenous protein breakdown in the absence of an exogenous source of nitrogen (Heald and Oxford, 1953), and ingest and digest rumen bacteria (Coleman, 1980). Many entodiniomorphs, including *Entodinium caudatum* (Abou Akkada and Howard, 1962; Onodera and Kandatsu, 1970), *Entodinium simplex* (Lockwood *et al.*, 1988), *Polyplastron multivesiculatum* (Lockwood *et al.*, 1988), *Eudiplodinium medium* (Naga and el-Shazly, 1968) and *Ophryoscolex* spp. (Williams *et al.*, 1961; Mah and Hungate, 1965; Lockwood *et al.*, 1988), are also proteolytic. Cell-free extracts of 14 individual entodiniomorphid species hydrolysed leaf fraction 1 protein, with *E. caudatum* and *E. simplex* having the highest activity and the cellulolytic species the lowest (Coleman, 1983). The optimum pH of proteolysis in cell extracts of *E. caudatum* and *Eudiplodinium maggii* was 3.2 with fraction 1 protein or casein as substrate (Coleman, 1983). However, maximal breakdown of casein was also reported to occur at pH 6.5–7.0 with extracts of *E. caudatum* (Abou Akkada and Howard, 1962) and the pH optimum for azocasein digestion by two species of holotrichs and four entodiniomorphs was between 4 and 5 (Lockwood *et al.*, 1988). Inhibition studies suggest that the main activities are thiol (cysteine) proteases (Coleman, 1983;

Lockwood *et al.*, 1988), although partial inhibition by pepstatin indicated the presence of some carboxyl (aspartic) types of enzyme (Coleman, 1983). Trypsin-like specificity was also detected (Abou Akkada and Howard, 1962). Patterns of SDS-PAGE zymograms suggest that the enzymes in the different protozoal species, even in the holotrichs, are different (Lockwood *et al.*, 1988).

Mixed intact and autolysed protozoa had fairly similar types of activity to those observed with individual species. Cysteine proteases were most prevalent (Forsberg *et al.*, 1984; Nagasawa *et al.*, 1992), and aspartate protease activity was also found in one of these studies (Forsberg *et al.*, 1984). Mixed protozoa had a much higher aminopeptidase activity than rumen bacteria and a slightly higher trypsin-like activity (Prins *et al.*, 1983; Forsberg *et al.*, 1984), which is consistent with these activities being lower in the rumen of ciliate-free sheep than in faunated animals (Wallace *et al.*, 1987*b*). Nagasawa *et al.* (1992) found that the exopeptidase activity of mixed protozoa was insensitive to bestatin and concluded that exopeptidase activity was different from aminopeptidase B and leucine aminopeptidase. Maximum proteolytic activity of intact mixed protozoa occurred at pH 5.9, and of sonicated organisms at pH 5.8 (Forsberg *et al.*, 1984).

The role of ciliate protozoa in the digestion of soluble proteins is not entirely clear. Brock *et al.* (1982) estimated that rumen ciliates were, on a specific activity basis, one-tenth as active in azocasein breakdown as rumen bacteria. A similar conclusion was reached by Nugent and Mangan (1981), as the hydrolytic products of fraction 1 protein appeared first in the bacteria, and then only subsequently became associated with protozoa as predation occurred. The proteolytic activity of rumen fluid, with ^{14}C-labelled casein as substrate, was changed little by faunation (Wallace *et al.*, 1987*b*), again consistent with a minor role for protozoa in soluble-protein breakdown, and similar conclusions were reached by Hino and Russell (1987) using inhibitors of protozoal growth, and Ushida *et al.* (1991). If soluble proteins are coagulated, however, which can occur as the result of holotrich activities (Onodera and Yakiyama, 1990), the coagulated protein could then be used by the entodiniomorphs (Onodera and Kandatsu, 1970; Onodera, 1990). However, the main role of the protozoa is likely to be in the hydrolysis of particulate proteins of an appropriate particle size. Chloroplasts, for example, are avidly engulfed by protozoa (Mangan and West, 1977), and stained particles of casein have a similar fate (Abou Akkada, 1965), with both types of particle being rapidly degraded intracellularly. Naga and el-Shazly (1968) demonstrated that the precise size of protein particles was important to *Eudiplodinium medium*. If particles were too small, their rate of hydrolysis decreased.

Even more significant is the predatory activity of protozoa against rumen bacteria, which is of enormous significance to bacterial protein turnover in the rumen. In the absence of protozoa, bacterial protein turnover varied

from 0.3% to 2.7% h^{-1} depending on species; in their presence, the rates increased to 2.4–3.7% h^{-1} (Wallace and McPherson, 1987). Clearly, therefore, the main role of the protozoa is not in the hydrolysis of exogenous soluble protein, but in metabolizing bacterial protein. Protozoa have lysozyme activity to effect the breakdown of bacterial cell walls, and also chitinase, which presumably may enable them to utilize fungal biomass in a similar way (Morgavi *et al.*, 1994).

Bacteria. Between 30% and 50% of the bacteria isolated from rumen fluid have proteolytic activity towards extracellular protein (Fulghum and Moore, 1963; Prins *et al.*, 1983). Representatives of most species have some activity, with the possible exception of the main cellulolytic bacteria, *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* and *R. albus*. *Ruminobacter amylophilus* is one of the most active proteolytic species isolated; since it is amylolytic, it would be expected to be of particular importance in starchy diets (Blackburn and Hobson, 1962; Abou Akkada and Blackburn, 1963). Proteolytic *Butyrivibrio fibrisolvens* can be the predominant proteolytic organisms isolated from some animals (Blackburn and Hobson, 1962; Fulghum and Moore, 1963; Hazlewood *et al.*, 1983; Wallace *et al.*, 1987*a*), and is probably enriched for when more resistant types of protein are present in the diet (Wallace *et al.*, 1987*a*). Probably the most numerous proteolytic bacterium is *Prevotella ruminicola*, proteolytic strains of which occur on both all-roughage and mixed roughage concentrate diets (Blackburn and Hobson, 1962; Abou Akkada and Blackburn, 1963; Hazlewood and Nugent, 1978; Wallace and Brammall, 1985). Indeed, *P. ruminicola* can comprise more than 60% of bacteria in the rumen (Van Gylswyk, 1990). However, proteolytic strains of *P. ruminicola* cannot be isolated from all animals (Hazlewood *et al.*, 1983). Bacteria with higher activity have also been isolated. These are often atypical organisms, like *Clostridium* (Hungate, 1966) or *Fusobacterium* (Wallace and Brammall, 1985). Other proteolytic bacteria more typical of predominant rumen species include species of the genera *Eubacterium*, *Lachnospira*, *Selenomonas* and *Succinivibrio* (Blackburn and Hobson, 1962; Abou Akkada and Blackburn, 1963; Fulghum and Moore, 1963; Wallace and Brammall, 1985). Proteolytic Gram-positive cocci were noted in early isolation experiments (Appleby, 1955; Blackburn and Hobson, 1960*a*, 1962), but *Streptococcus bovis* was not recognized as a potentially major proteolytic organism until more recently (Russell *et al.*, 1981; Hazlewood *et al.*, 1983; Wallace and Brammall, 1985). In the survey by Attwood and Reilly (1995), *S. bovis* isolates were particularly numerous among the highest-activity strains examined in New Zealand cattle grazing pasture; also present were some high-activity *B. fibrisolvens* strains, but 10 strains of a previously unrecognized proteolytic species, *Eubacterium budayi*, were present in a total of 43 high-activity isolates. Other proteolytic, facultatively anaerobic bacteria

isolated in earlier studies are probably of little importance in the mainly strictly anaerobic flora.

Most attention has been focused on the three species considered to be the major proteolytic organisms, namely *Ruminobacter amylophilus*, *B. fibrisolvens* and *P. ruminicola*. *Ruminobacter amylophilus* at first sight appears to be peculiar, because it does not require peptides or amino acids for growth (Hobson *et al.*, 1968; Jenkinson *et al.*, 1979). Even if these are available, ammonia remains the principal nitrogen source, with only a small proportion of cell nitrogen derived from labelled protein, peptides or amino acids (Hobson *et al.*, 1968; Hullah and Blackburn, 1971). Furthermore, protease is produced in medium devoid of proteins or amino acids (Blackburn, 1968*a*). The retention of such an apparently gratuitous protease activity in a competitive ecosystem like the rumen prompted Cotta and Hespell (1986*a*) to suggest that its function was not a nutritional one, but rather to break down structural proteins within cereal particles, thereby exposing starch granules to amylolytic attack. In contrast, both *P. ruminicola* and *B. fibrisolvens* can grow in a medium containing protein as the sole source of nitrogen (Hazlewood and Nugent, 1978; Wallace and Brammall, 1985; Cotta and Hespell, 1986*b*).

The production of protease activity seems to be only loosely regulated in these bacteria. Blackburn (1968*a*) concluded that the composition of the growth medium in batch cultures had little influence on the expression of protease activity by *Ruminobacter amylophilus*, whereas growth rate had a small effect, with more activity tending to be produced at lower growth rates (Henderson *et al.*, 1969). In contrast, the activity produced by *B. fibrisolvens* with different carbon sources increased as the growth rate elicited by each compound increased, the activity at $0.62\,h^{-1}$ on fructose as carbon source being double that at $0.22\,h^{-1}$ on xylose (Cotta and Hespell, 1986*b*). High concentrations ($20\,g\,l^{-1}$) of amino acids or peptides in the medium severely repressed activity, while amino acids at $1\,g\,l^{-1}$ caused threefold stimulation relative to that expressed with casein (Cotta and Hespell, 1986*b*). Similar studies of the regulation of protease activity in *P. ruminicola* have not been done, although Hazlewood *et al.* (1981) found that the activity expressed depended on the protein used as growth substrate. Only 37% of the proteolytic activity found in cultures containing fraction 1 protein occurred when albumin was the substrate. The protease activity of other rumen bacteria was affected little by different sources of nitrogen in the medium (Wallace and Brammall, 1985).

Fractionation of rumen fluid has shown clearly that most proteolytic activity is cell-associated (Blackburn and Hobson, 1960b; Nugent and Mangan, 1981; Brock *et al.*, 1982; Kopecny and Wallace, 1982; Prins *et al.*, 1983). Indeed, the soluble activity that can be found in the cell-free supernatant fluid may have been largely displaced from the main site of enzyme activity, namely exocellular polysaccharide capsular material

(Kopecny and Wallace, 1982). Cell-associated proteases can be liberated by gentle shaking, treatment in a Waring Blendor or extraction with Triton X-100 (Kopecny and Wallace, 1982; Prins *et al.*, 1983). This cell surface location results in a mechanism of proteolysis whereby the substrate protein adsorbs rapidly and irreversibly to rumen bacteria as an integral part of the process (Nugent and Mangan, 1981; Wallace, 1985*a*). Within individual species, protease activity is substantially cell-bound in *Ruminobacter amylophilus* (Blackburn, 1968*a*; Blackburn and Hullah, 1974), *P. ruminicola* (Hazlewood *et al.*, 1981; Wallace and Brammall, 1985), *Eubacterium* sp. (Wallace and Brammall, 1985) and some low-activity isolates of *B. fibrisolvens*, *Selenomonas ruminantium* and *Streptococcus bovis* (Wallace and Brammall, 1985). The activities of both *P. ruminicola* and *Ruminobacter amylophilus* remain almost entirely cell-associated during growth, and are released into the medium largely as the result of autolysis in stationary phase (Lesk and Blackburn, 1971; Hazlewood *et al.*, 1981). In contrast, high-activity strains of *B. fibrisolvens* produce an activity that is always extracellular (Wallace and Brammall, 1985; Cotta and Hespell, 1986*b*).

Proteolytic activity has a broad pH optimum around approximately pH 5.5–7.0 in rumen fluid (Blackburn and Hobson, 1960b), mixed rumen bacteria (Kopecny and Wallace, 1982) and extracted bacterial enzymes (Kopecny and Wallace, 1982). *B. fibrisolvens* protease has a similar pH optimum (Cotta and Hespell, 1986*b*), while *Ruminobacter amylophilus*, pH range 5.5–9.5 for the soluble enzyme (Blackburn, 1968*b*), and 4.5–12.0 for the cell-associated enzyme (Lesk and Blackburn, 1971), and *P. ruminicola*, pH 5.9–8.2 (Hazlewood *et al.*, 1981), were active at the same and higher pH values.

It can be concluded from the effects of protease inhibitors that the predominant type of protease present in rumen contents (Wallace, 1984), mixed rumen bacteria (Brock *et al.*, 1982; Kopecny and Wallace, 1982; Wallace and Brammall, 1985) and extracted capsular enzymes (Kopecny and Wallace, 1982; Prins *et al.*, 1983) is a cysteine protease type, sensitive to *p*-chloromercuribenzoate (PCMB). Other types of activity are also present, but are more variable. These include phenylmethylsulphonyl fluoride (PMSF)-sensitive protease (present at 0–41% of total activity), metalloprotease (9–30%) and aspartic protease (2–15%) (Brock *et al.*, 1982; Kopecny and Wallace, 1982; Prins *et al.*, 1983; Wallace, 1984; Wallace and Brammall, 1985). Among the proteolytic species that have been examined, *P. ruminicola* produces an activity most similar to that of rumen contents. PCMB was highly inhibitory (56–89%), and serine protease inhibitors reduced the activity by 21–43% (Hazlewood and Edwards, 1981; Wallace and Brammall, 1985). *P. ruminicola* was more sensitive to EDTA than mixed rumen bacteria (Hazlewood and Edwards, 1981; Wallace and Brammall, 1985), but the effects of chelators may be complex and they do not always indicate a metalloprotease (Hazlewood and Edwards, 1981). *Ruminobacter*

amylophilus had, in contrast, a predominantly serine protease activity (Wallace and Brammall, 1985), as did most strains of *B. fibrisolvens* (Wallace and Brammall, 1985; Cotta and Hespell, 1986*b*). Indeed, all 10 zymogram bands of activity in supernatant fluids from *B. fibrisolvens* had an activity that was inhibited by PMSF (Strydom *et al.*, 1986). A low-activity strain of *B. fibrisolvens* had an activity that was inhibited only 12% (cf. >90% for other strains) by PMSF and 93% by PCMB (Wallace and Brammall, 1985). *Streptococcus bovis* and a proteolytic *Eubacterium* sp. also had PMSF-sensitive activity, whereas the low activity of *Selenomonas ruminantium* was insensitive to PMSF, but was inhibited by PCMB and to a lesser extent by EDTA (Wallace and Brammall, 1985).

Not surprisingly in view of the different proteolytic species present in different diets, the type of protease present is influenced by animal diet (Prins *et al.*, 1983; Wallace and Brammall, 1985). Indeed, a cow on a hay diet had no serine protease in its rumen bacterial fraction (Prins *et al.*, 1983).

The flora associated with the rumen wall also has protease activity, of a slightly different type from that of rumen contents (Wallace, 1984). The bacteria have a very high specific activity, in keeping with their evident active digestion of epithelial tissue, but the numbers of these organisms are low relative to total rumen contents, and their contribution to proteolysis will be limited to their effect at the rumen wall (Dinsdale *et al.*, 1980; Wallace, 1984).

The proteases of rumen bacteria have several different kinds of specificity. Among mixed rumen bacteria, work with synthetic substances has shown mainly trypsin-like and leucine aminopeptidase activities, as judged by the hydroysis of benzoyl-arginine *p*-nitroanilide (BAPNA) and leucine *p*-nitroanilide (LNA), respectively (Brock *et al.*, 1982; Prins *et al.*, 1983; Wallace and Kopecny, 1983) and by the inhibition of proteolysis by the trypsin substrate analogues *N*-tosyl-L-lysine chloromethyl ketone (TLCK) (Brock *et al.*, 1982; Wallace and Kopecny, 1983) and *N*-tosyl-L-lysine chloromethane (Prins *et al.*, 1983). Prins *et al.* (1983) and Wallace and Kopecny (1983), using synthetic chymotrypsin substrates, found that chymotrypsin-like activity was present, but low, in rumen bacteria and extracted bacterial cell-envelope enzymes. Chymotrypsin inhibitors had little effect (Prins *et al.*, 1983; Wallace and Brammall, 1985). In contrast, Brock *et al.* (1982) obtained 21% inhibition of bacterial proteolysis by *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), and a substantial activity against the chymotrypsin substrate *N*-3-(carboxypropionyl)-L-phenylalanine *p*-nitroanilide. The latter activity was, however, measured with sonicated bacteria and therefore included intracellular activity not usually involved in the breakdown of exogenous protein. Carboxypeptidase activity was of a similar magnitude to chymotrypsin-like activity (Prins *et al.*, 1983; Wallace and Kopecny, 1983), i.e. of less importance than the two main activities of trypsin and leucine aminopeptidase-like specificity.

None of the proteolytic bacteria isolated from the rumen has a particularly distinctive activity, except possibly *Streptococcus bovis*, which has an exceptionally high leucine aminopeptidase activity (Wallace and Brammall, 1985). The cell-associated activity of *Ruminobacter amylophilus* (Blackburn, 1968*b*) and the soluble protease released in late stationary phase (Lesk and Blackburn, 1971) were active against trypsin substrates and inhibited by trypsin substrate analogues. Some aminopeptidase (Blackburn, 1968*b*) activity was also observed. *P. ruminicola* has no activity against BAPNA, although it was inhibited slightly by TLCK (Wallace and Brammall, 1985), leupeptin and soybean trypsin inhibitor (Hazlewood and Edwards, 1981), indicating some trypsin-like activity. Chymostatin was more inhibitory, giving 38–63% inhibition (Hazlewood and Edwards, 1981), so the specificity may be more of a chymotrypsin type. High-activity *Butyrivibrio* spp. also did not hydrolyse BAPNA (Wallace and Brammall, 1985) and were not significantly inhibited by TLCK or TPCK (Wallace and Brammall, 1985; Cotta and Hespell, 1986b) or trypsin inhibitor I-S (Cotta and Hespell, 1986*b*). A low-activity isolate differed in that TLCK and TPCK caused 32% and 28% inhibition (Wallace and Brammall, 1985). The low activity of *Selenomonas ruminantium* was significantly inhibited by TPCK, indicative of chymotrypsin-like activity, and that of a *Eubacterium* sp. was active against LNA, indicating a leucine aminopeptidase activity.

What bacterial species is therefore most important in protein digestion in the rumen? Since bacteria can interact synergistically with each other in the degradation of protein, there may not be a simple answer. Cooperative proteolysis has been observed between *P. ruminicola* and *Selenomonas ruminantium*, *Streptococcus bovis* and *Selenomonas ruminantium*, and some other pairs of species (Wallace, 1985*b*). The interaction between *Streptococcus bovis* and *Selenomonas ruminantium* enabled rapid growth on a medium containing casein as sole nitrogen source, in which either species inoculated alone grew poorly. Presumably, more complex mixtures of species would give even better cooperativity, depending on the type of proteolytic specificity possessed by each and required in the breakdown of the protein. No single species should be judged in isolation, therefore. There is also variability between diets and even between animals. Direct zymogram analysis of the proteinases present in the rumens of different sheep on the same and different diets illustrates just how variable the proteolytic population can be (Figure 7.2). Sometimes the same band of protease activity can be detected in different samples. However, the major bands of activity are rarely the same. Hence, there is an intrinsic variability that probably reflects the many different species possessing proteolytic activity that can from time to time dominate the flora.

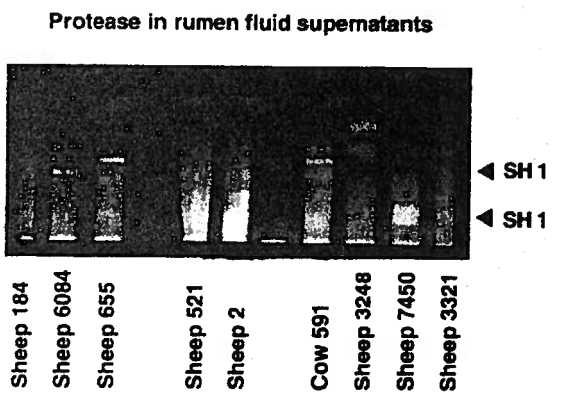

Figure 7.2 Gelatin-PAGE zymogram of extracellular protease activities in different samples of rumen fluid. Arrows refer to the location of protease bands from *B. fibrisolvens* SHI, of approximate M_r 83 500 and 133 000. Sheep 184, 521 and 2 received a hay diet; sheep 6084, 3248, 7450 and 3321 were fed on dried grass cubes; sheep 655 and cow 591 received mixed diets consisting of 2:1 hay + cereal concentrate and equal parts of hay, grass cubes and concentrate respectively.

Fungi. Reports of proteolytic activity in anaerobic rumen fungi are conflicting to some extent. Some studies have measured and characterized various aspects of fungal proteolytic activity, while others have failed to find significant activity. Proteolytic activity in rumen fungi was first reported in an isolate of *Neocallimastix frontalis* (Wallace and Joblin, 1985). This was an extracellular activity which, from its inhibition by EDTA and 1,10-phenanthroline (the former inhibition was reversed by the addition of divalent cations, particularly Zn^{2+}), and its sensitivity to TLCK, was concluded to be a zinc metalloprotease with a trypsin-like specificity. Other fungal isolates of different morphology also had metalloprotease activity (Wallace, unpublished observations). A *Neocallimastix* sp. and a *Piromyces* sp. were also found to have metalloprotease activity (Asao *et al.*, 1993), and in a study examining nutritional factors affecting fungal proteolytic activity, all species examined had proteolytic activity, with *Orpinomyces joyonii* producing the highest specific activity (Yanke *et al.*, 1993). Fungal proteolytic activity was highest on corn compared to wheat or barley, prompting speculation about the role of fungi in breaking down the protein matrix of corn (Yanke *et al.*, 1993). In contrast, Michel *et al.* (1993) found little proteolytic activity in their fungal isolates, and experiments with gnotobiotic lambs suggested that fungal protease made a minor contribution to ruminal proteolysis (Bonnemoy *et al.*, 1993). This conclusion is

probably valid. Although fungi competed successfully with bacteria *in vitro* (Wallace and Munro, 1986), the low metalloproteinase activity of rumen contents suggests that they play a minor role *in vivo*.

7.2.2 *Peptide breakdown*

Hydrolysis of proteins by rumen microbial enzymes releases oligopeptides, which are then broken down in turn to smaller peptides and, finally, to amino acids. Peptide breakdown to amino acids must occur for the amino acids to be incorporated into microbial protein, and when there is sufficient energy available to fuel biosynthesis, the amino acids released will be incorporated and peptide breakdown would not be considered to be a major inefficiency in fermentation. However, when energy is unavailable, or when the rate of peptide breakdown exceeds the rate at which it can be assimilated, peptide catabolism contributes to excessive ammonia production and poor nitrogen retention.

Most reports of the uptake of ^{14}C-labelled amino acids and peptides until recently indicated that the mixed microbial population preferentially incorporates peptides rather than free amino acids (Wright, 1967; Prins *et al.*, 1979; Cooper and Ling, 1985). Results on the preferred size of peptides were inconsistent (Wright, 1967; Cooper and Ling, 1985; Armstead and Ling, 1993), but these differences appear to be mainly methodological. This apparent preference for peptides appeared to be reinforced by pure culture studies with *Prevotella ruminicola*, which showed that larger peptides were preferred for growth (Pittman and Bryant, 1964) and that peptides up to a molecular weight of 2000 were taken up much more rapidly than free amino acids (Pittman *et al.*, 1967). However, Armstead and Ling (1993) found that amino acids were preferred over peptides in rumen fluids from some sheep, much more so than in others, and they suggested that there may be a dependence on diet. Recent experiments with individual bacterial species tend to support this view. Amino acid transport was much more predominant than peptide transport in *Streptococcus bovis* (Westlake and Mackie, 1990), and Ling and Armstead (1995) found that free amino acids were the preferred form of amino acids incorporated by *Streptococcus bovis*, *Selenomonas ruminantium*, *Fibrobacter succinogenes* and *Anaerovibrio lipolytica*, while peptides were preferred only by *P. ruminicola*. Thus the observed preference for peptides by the mixed population in most studies must presumably reflect large numbers of *P. ruminicola* in the microflora. As described already, *P. ruminicola* can comprise >60% of the total flora in sheep receiving grass silage (Van Gylswyk, 1990). In other studies where an amino acids preference was exhibited, presumably numbers of *P. ruminicola* were much lower.

Peptide breakdown in excess of the rate at which the products are incor-

porated into microbial protein is undesirable and inefficient. Different peptides are broken down at different rates by the mixed ruminal population. Chen *et al.* (1987*a*) observed that an isopropanol extract of Trypticase, containing a high proportion of hydrophobic amino acid residues, was broken down more slowly than the corresponding water-soluble peptides, and concluded that the hydrophobicity of peptides determined their rate of breakdown. No evidence of such a relation was found when the metabolism of individual peptides was studied, however; indeed Phe_4 was among the most rapidly degraded peptides (Wallace and McKain, 1989*a*; Wallace, 1996). A recent study of the degradation of a soya protein hydrolysate also showed no preference for hydrophilic peptides (Depardon *et al.*, 1995). The main features of the amino acid sequence that dispose some peptides to be more resistant than others to degradation appear to be Gly-Gly, Pro-X or X-Pro residues at the N-terminus or >1 acidic residues in the peptide (Broderick *et al.*, 1988; Wallace *et al.*, 1990*a*; Yang and Russell, 1992; Wallace, 1996). Peptides which survive for a prolonged time in rumen fluid tend to be enriched in these amino acids (Wallace *et al.*, 1993*a*). The great majority of peptidase activity in the rumen is aminopeptidase (Wallace *et al.*, 1990*a*) a property that confers on N-terminally blocked peptides a high degree of resistance to ruminal degradation (Wallace, 1992*a*; Wallace *et al.*, 1993*b*). The peptides present in rumen fluid several hours after feeding also had a lower than expected response to the peptide reagent, fluorescamine, which requires a free $-NH_2$ group to react (Wallace and McKain, 1990). Hence some of these peptides may survive degradation because they are naturally N-formylated or -acetylated. Other peptides are broken down extremely rapidly (Wallace, 1996), and it is the breakdown of these peptides that is of greatest concern.

The predominant aminopeptidase activity found in mixed ruminal bacteria cleaves dipeptides rather than single amino acids from the peptide chain (Wallace *et al.*, 1990*a*, 1993*c*; Depardon *et al.*, 1995). Enzymes of this nature are classified as dipeptidyl peptidases (Webb, 1992). Enzyme activities against synthetic peptidase substrates showed that dipeptidyl–X substrates were much more rapidly degraded than amino acyl–X substrates (Wallace and McKain, 1989*b*; Wallace, 1996). Thus peptide breakdown in rumen fluid is a two-step process, dipeptidyl peptidases releasing dipeptides from oligopeptides, followed by separate dipeptidases which cleave the resulting dipeptides to amino acids. Remarkably, the only common bacterial species that possesses high dipeptidyl peptidase activity is *P. ruminicola* (Wallace and McKain, 1991; McKain *et al.*, 1992; Wallace *et al.*, 1997). *P. ruminicola*, like the mixed rumen population, had low aminopeptidase activity against amino acyl–*p*-nitroanilide substrates but high activity against dipeptidyl–*p*-nitroanilide substrates; it also cleaved dipeptides from longer peptides as the first step in peptide hydrolysis (Wallace *et al.*, 1993*c*). Once again, the pattern of peptide breakdown would be expected to change with diet and its

influence on numbers of *P. ruminicola*. If organisms such as *Streptococcus bovis* were to prevail, their leucine aminopeptidase activity would predominate and amino acids might be cleaved singly from the peptide chain rather than in pairs (Russell and Robinson, 1984; Wallace and Brammall, 1985). In contrast to the limited occurrence of dipeptidyl aminopeptidase, many species of protozoa and bacteria have dipeptidase activity. Among the bacteria, *P. ruminicola* had activity against a wide range of dipeptides, while *Megasphaera elsdenii* also had a high activity (Wallace and McKain, 1991).

Ciliate protozoa have exopeptidase activities (Nagasawa *et al.*, 1992) and produce amino acids from protein in incubations *in vitro* (Coleman and Laurie, 1974; Ueda *et al.*, 1975; Coleman and Sandford, 1979*a*). *Diploplastron affine*, *Enoploplastron triloricatum*, *Ostracodinium abtusum dilobum*, *Diplodinium affine* and *Diplodinium anisacanthum* use plant protein for growth (Coleman and Reynolds, 1982), and virtually all entodiniomorphs and holotrichs digest engulfed bacterial protein (Coleman, 1980). The protozoa making the greatest contribution are probably, due to their generally higher numbers, the small entodiniomorphs (Coleman, 1980; Coleman, 1983; Wallace and McPherson, 1987). It was established by comparing the specific activities of mixed rumen protozoa and mixed bacteria prepared from rumen fluid that bacteria were mainly responsible for the breakdown of larger peptides (Newbold *et al.*, 1989; Wallace *et al.*, 1990*b*). However, the ciliates had a particularly high dipeptidase activity (Newbold *et al.*, 1989; Wallace *et al.*, 1996), and this may be their main contribution to peptide metabolism. However, this does not mean that defaunation would result in decreased dipeptide breakdown; the dipeptidase activity of ciliate-free sheep was the same as that of faunated animals (Wallace *et al.*, 1987*b*), suggesting that bacteria can occupy the same niche as protozoa in this respect.

Rumen fungi also possess aminopeptidase but not carboxypeptidase activity (Michel *et al.*, 1993), but the significance of fungal peptidase activity, or of peptides in fungal metabolism and growth, has not been investigated.

Whether or not peptides accumulate in rumen fluid depends on the nature of the protein and probably also the nature of the diet. Nugent and Mangan (1981) did not detect peptide accumulation following the hydrolysis of ^{14}C-labelled fraction 1 protein *in vitro*, but Chen *et al.* (1987*b*) reported significant peptide accumulation from the breakdown of soybean meal in the bovine rumen. Less accumulated when the soybean meal was heat-treated, but the quantity of peptides flowing from the rumen was still significant. Broderick and Wallace (1988) found that peptides accumulated in the sheep rumen when casein was used as a protein supplement, but they were not observed when the supplement was the more slowly degraded egg albumin. Thus proteins that are rapidly hydrolysed in the rumen could lead to the build-up of peptides in rumen fluid, and the flow of intact peptides from the rumen could be significant. However, for many proteins the rate of

utilization of peptides probably exceeds their rate of production. Precise analysis of peptides by the free amino acids released from protein-free rumen fluid during acid hydrolysis is essential for a valid estimate of peptide concentration to be made (Wallace and McKain, 1991; Williams and Cockburn, 1991). All chemical methods have drawbacks (Wallace and McKain, 1991).

Slowing peptide breakdown would help to decrease the flux of protein nitrogen to ammonia in the rumen and therefore help to increase the efficiency of nitrogen retention by the ruminant animal. Only a few means of inhibiting peptide breakdown have so far been investigated. Ionophores such as monensin and tetronasin have been found, among their many other effects, to cause peptides to accumulate in rumen fluid or *in vitro* fermentations when the microbial population has been treated for a prolonged time (Whetstone *et al.*, 1981; Newbold *et al.*, 1990; Wallace, 1992*b*). However, the acute addition of monensin or tetronasin to rumen fluid *in vitro* had no effect on the rate of peptide breakdown (Wallace *et al.*, 1990*c*), suggesting that adaptation was required for the inhibitory effect on peptide metabolism to take place. It is possible that the species present in the rumen microbial population alter in response to ionophores, causing a change in peptide metabolism. Alternatively, *P. ruminicola* decreases its membrane permeability upon prolonged exposure to ionophores, which slows its rate of peptide metabolism (Newbold *et al.*, 1992), and so may alter the peptidolytic properties of the whole population.

The low carboxypeptidase activity in the rumen (Wallace *et al.*, 1990*a*) means that peptides can be blocked effectively from degradation by treatment with acetic anhydride or similar anhydrides (Wallace, 1992*a*; Wallace *et al.*, 1993*b*). Such *N*-acetylated peptides are, unlike their unmodified form, stable in rumen contents for several hours. Acetylation could therefore convert rapidly degraded peptides in food or other byproducts to undegraded dietary protein (UDP). Alternatively, it could be used to deliver defined peptides to the abomasum, perhaps as a means of enhancing the flow of defined amino acids to the small intestine. A different method for slowing peptide breakdown was investigated following the discovery that the dipeptidase activity of *P. ruminicola* was inhibited strongly by 1,10-phenanthroline, a chelator of divalent transition metal ions (Wallace *et al.*, 1995*a*). It emerged that protozoal and other bacterial dipeptidases were also sensitive to 1,10-phenanthroline and that Trypticase breakdown could be inhibited up to 71% by 1,10-phenanthroline (Wallace *et al.*, 1996). The effect was due partly to inhibition of dipeptidase, but also of dipeptidyl peptidase activity, which also has a metal ion requirement (Madeira and Morrison, 1995; Wallace *et al.*, 1996). Heavy metal ions and analogues of 1,10-phenanthroline inhibited the dipeptidase of *P. ruminicola*, but the latter group all had chelating activity and would be likely to affect other activities of rumen microorganisms (Wallace and McKain, 1996). A more

specific inhibitor, possibly of dipeptidyl peptidases, would be required for nutritional use.

7.2.3 Amino acid breakdown

Metabolism of the amino acids themselves is the next stage in the metabolism of most of the constituents of dietary protein. As our knowledge of the importance of peptide metabolism in microbial growth and ammonia production improves, it becomes clearer that studying the metabolism of extracellular free amino acids, particularly single amino acids, may be misleading if we are interested in amino acids that originate from dietary protein. The rates of entry of peptide-bound amino acids into cells may be quite different from the rates of entry of the corresponding free acids (Prins *et al.*, 1979). Furthermore, experiments with high concentrations of amino acids in incubations *in vitro*, such as the 10 mM used by Lewis and Emery (1962), may be misleading because concentrations *in vivo* are much lower.

There is little free amino acid in rumen fluid, and what there is occurs for the most part intracellularly (Wright and Hungate, 1967; Wallace, 1979). Glutamate, for example, is present almost totally as intracellular pools (Wright and Hungate, 1967; Wallace, 1979). Even 1 h after feeding, when there is a major increase in the α-amino nitrogen, the free amino acid content of rumen fluid is quite low (Leibholz, 1969). The extent of accumulation varies with diet, with the highest concentrations observed after feeding alfalfa hay (Leibholz, 1969). Broderick and Wallace (1988) demonstrated that amino acids accumulate from rapidly degraded, but not slowly degraded, protein. The overflow of free amino acids during the hydrolysis of fraction 1 protein was low (Nugent and Mangan, 1981). Hino and Russell (1985) compared the deaminase activity of intact microorganisms and cell extracts, and concluded that the high capacity for deamination implied that the rate of uptake of peptides or amino acids into cells might limit the rate of ammonia production.

The observed rates of amino acid deamination at so-called 'physiological' concentrations are indeed rapid. Of the amino acids essential to the animal, lysine, phenylalanine, leucine and isoleucine are broken down at 0.2–0.3 mmol h^{-1}, while arginine and threonine are more labile (0.5–0.9 mmol h^{-1}) and valine and methionine are most stable (0.10–0.14 mmol h^{-1}) (Chalupa, 1976). Experiments with non-essential amino acids suggest that they are metabolized at least as rapidly as essential amino acids (Broderick and Balthrop, 1979). However, methionine is one of the most slowly degraded amino acids, to such an extent that it has been suggested that protection may not be necessary to deliver substantial amounts of undegraded methionine to the abomasum, if the quantity provided is sufficient (Cottle and Velle, 1989).

The carbon skeletons arising from deamination give rise to a variety of volatile fatty acid (VFA) products, as reviewed by Blackburn (1965) and Allison (1970). Presumably the purpose of this metabolism is to provide energy for the microorganisms, as otherwise there would be no advantage in their having the activity. There is some evidence of Stickland-like reactions in rumen fluid, whereby pairs of amino acids are metabolized and provide energy via coupled oxidation and reduction (Barker, 1981), because sodium arsenite caused a 70% inhibition of the metabolism of the amino acids in an acid hydrolysate of casein (Broderick and Balthrop, 1979). However, other experiments with pairs of amino acids and rumen microorganisms were inconclusive as to whether these reactions are significant in the rumen (Lewis and Emery, 1962; Van den Hende *et al.*, 1963). Energy is presumably also produced from the metabolic sequence leading to VFA production from branched-chain amino acids (Harwood and Canale-Parola, 1981).

Despite the energy potentially available from the deamination of amino acids, neither mixed rumen bacteria (Russell *et al.*, 1983) nor pure cultures of *P. ruminicola* (Russell, 1983) or *M. elsdenii* (Russell and Baldwin, 1979; Wallace, 1986*a*) gave much higher yields as a result of amino acid fermentation. It was suggested from the pure-culture work that the energy produced would contribute only to the maintenance energy of the bacteria. Branched-chain VFA formation by *M. elsdenii* occurred during stationary phase, in low-energy conditions, rather than during growth (Allison, 1978), again consistent with their role in maintenance. However, there is now evidence that a relatively small population of atypical bacteria may be responsible for a significant amount of the amino acid deamination which occurs in the mixed population.

Russell and his colleagues at Cornell University calculated that the predominant rumen bacteria which had been identified as ammonia producers by Bladen *et al.* (1961) did not have sufficient activity to account for observed rates of ammonia production by the mixed population in their cattle, and isolated from these animals a group of bacteria which possessed a specific activity of ammonia production that was an order of magnitude greater than that of the other species (Chen and Russell, 1988, 1989; Russell *et al.*, 1988, 1991). These bacteria were asaccharolytic, relying on the fermentation of amino acids for growth. Moreover, they, unlike the others, were highly sensitive to monensin, and since ammonia concentrations are lower when ruminants receive this dietary ionophore, it was deduced that they must be significant ammonia producers *in vivo*. The species isolated, *Peptostreptococcus anaerobius*, *Clostridium sticklandii* and *Clostridium aminophilum* (Paster *et al.*, 1993), were atypical of the main ruminal species, although a large number of clostridia have been isolated from the rumen over the years (Chapter 2). These bacteria, and also the mimosine degrader, *Synergistes jonesii* (Allison *et al.*, 1992; McSweeney *et al.*, 1993), did not

HIGH NUMBERS LOW ACTIVITY	LOW NUMBERS HIGH ACTIVITY
Butyrivibrio fibrisolvens *Megasphaera elsdenii* *Prevotella ruminicola* *Selenomonas ruminantium* *Streptococcus bovis*	*Clostridium aminophilum* *Clostridium sticklandii* *Peptostreptococcus anaerobius*
$>10^9$ per ml	10^7 per ml
10-20 nmol NH_3 min^{-1} (mg protein)$^{-1}$	300 nmol NH_3 min^{-1} (mg protein)$^{-1}$
Mainly monensin-resistant	Monensin-sensitive

Figure 7.3 A summary of the properties of ammonia-producing bacteria from the rumen. Reproduced from Wallace (1996), with permission.

ferment sugars but used amino acids as their main source of carbon and energy as well as a nitrogen source. Thus amino acid deamination could be carried out predominantly by numerically abundant bacteria each having low activity, or by relatively few species each with exceptionally high deaminative activity (Figure 7.3). The answer probably lies between the two and will be heavily dependent on diet. rRNA probing will help to assess the population sizes of the amino acid fermenters (Krause and Russell, 1996). Traditional culture methods revealed that the numbers of these bacteria were very low in rumen fluid from sheep with low rates of ammonia production (Wallace *et al.*, 1995*b*).

The disposal of reducing equivalents can prove to be problematic in anaerobic ecosystems. In the rumen, disposal is usually achieved by methanogenesis. When methane formation was inhibited by carbon monoxide, which inhibits bacterial hydrogenases, the fermentation stoichiometry switched to a higher propionate production (Russell and Jeraci, 1984). A more surprising secondary effect was that ammonia production declined, primarily as a consequence of inhibition of branched-chain amino acid fermentation (Russell and Jeraci, 1984; Russell and Martin, 1984). In cell extracts of rumen bacteria, the $NADH/NAD^+$ ratio was an important effector of branched-chain amino acid fermentation, with NAD^+ being essential as an electron acceptor (Hino and Russell, 1985). Thus, when hydrogenase was inhibited by carbon monoxide, the $NADH/NAD^+$ ratio increased and amino acid deamination declined (Hino and Russell, 1985). The feed ionophores, monensin and lasalocid, also inhibited both methanogenesis and deamination, but by a mechanism that could not be explained solely by their effect on hydrogenase activity (Russell and Martin, 1984).

Ciliate protozoa seem to have a significant role in deamination. Most species of protozoa produce ammonia from protein or amino acids (Abou Akkada, 1965; Allison, 1970; Coleman, 1980; Williams, 1986). Indeed, the

specific activity of mixed protozoa appeared to be approximately three times that of bacteria (Hino and Russell, 1985). Ammonia concentrations in faunated sheep can be about twice those in ciliate-free animals (Eadie and Gill, 1971) and deaminase activities were higher in faunated sheep, particularly when only small entodinia were present (Wallace *et al.*, 1987*b*). Ciliates deaminate a relatively small number of amino acids, the ammonia being produced mainly from glutamine, asparagine, citrulline, arginine and ornithine, and not from glutamate, asparate or histidine (Onodera *et al.*, 1983; Onodera and Goto, 1990). The highest ammonia-producing activity of the ciliates may therefore be principally amidase, as suggested by Abou Akkada and Howard (1962). Products other than ammonia arising from protozoal breakdown of amino acids include 2-oxobutyric and 2-aminobutyric acids, from threonine and methionine (Onodera and Migita, 1985), pipecolic acid from lysine (Onodera and Kandatsu, 1972), δ-aminovaleric acid from proline (Onodera *et al.*, 1983) and methionine sulphoxide (Onodera and Takei, 1986). The main products of amino acid catabolism by protozoa are, however, similar to those of the bacteria, i.e. short- and branched-chain VFAs (Allison, 1970; Coleman, 1980). Exogenously supplied amino acids were not catabolized by *Isotricha* spp. (Wallis and Coleman, 1967; Harmeyer, 1971), but deamination of amino acids has been observed with *E. caudatum* (Coleman, 1967; Wakita and Hoshino, 1975), which released pipecolic acid (Onodera and Kandatsu, 1969), and with *E. ecaudatum caudatum* (Coleman and Laurie, 1974). Transport limits the rate of metabolism of exogenous amino acids by ciliates, however, and rates of deamination of amino acids in the protein of intact bacteria may prove a better measurement of protozoal deaminative activity (Onodera and Takashima, 1989). Williams and Coleman (1992) compared the growth rates of several species of protozoa on bacterial protein and on exogenous free amino acids; only *Polyplastron multivesiculatum* grew at a similar rate on both substrates, while other species grew much faster in the presence of bacteria.

Different bacterial species utilize different spectra of amino acids during growth (Scheifinger *et al.*, 1976), which may cause different patterns of amino acid deamination in different animals and in animals on different diets, depending on the bacterial populations present. Some organisms, such as species of *Megasphaera*, *Streptococcus* and *Eubacterium*, removed a substantial proportion of all amino acids present in a mixture in the medium, while *Butyrivibrio* and *Selenomonas* were more selective. Methionine was actually produced by three out of seven isolates tested (Scheifinger *et al.*, 1976). As is found with the mixed population, the pattern of amino acid utilization by individual species is somewhat different when amino acids are present in peptides rather than in the free form (Cotta and Russell, 1982; Wallace, 1986*a*). The degradation products of amino acids in pure cultures are principally short- and branched-chain VFAs (Blackburn, 1965; Allison, 1970). However, the product of tryptophan breakdown in the rumen,

skatole (3-methylindole), is of interest as it causes bovine pulmonary emphysema (Carlson *et al.*, 1975). The skatole-producing organism was reported to be a *Lactobacillus* sp. which produces skatole by decarboxylation of indoleacetic acid (Yokoyama *et al.*, 1977; Yokoyama and Carlson, 1981). Presumably because lactobacilli are sensitive to this ionophore, monensin prevents the disease (Hammond *et al.*, 1978) and salinomycin inhibits skatole production (Onodera *et al.*, 1992).

The inhibition of amino acid degradation is an obvious objective for manipulation. Even if the amino acids were not to pass undegraded from the rumen, if they were to be incorporated directly into microbial protein rather than be degraded to ammonia and then resynthesized, the energy cost of resynthesis would be saved. Nutritional management to ensure the simultaneous availability of energy and amino acids to rumen fermentation is an obvious means of improving nutrient utilization (Sinclair *et al.*, 1995). As has already been mentioned, ionophores achieve partial inhibition of ammonia production, but this is not their primary effect. Diaryliodonium compounds were intended specifically as inhibitors of amino acid degradation (Chalupa, 1977, 1980) and were found to be successful in improving nitrogen retention *in vivo* (Chalupa *et al.*, 1976). The organism most sensitive to diphenyliodonium chloride was *P. ruminicola* (Wallace, 1986*b*), and the improved nitrogen retention due to inhibition of *P. ruminicola* is consistent with the proposed central role of this organism in protein, peptide and amino acid catabolism. Few chemicals will be entirely specific, however. Effects other than those on amino acid metabolism were seen with diaryliodonium compounds, including inhibition of methanogenesis (Chalupa, 1980). Deamination can be inhibited by hydrazine and similar

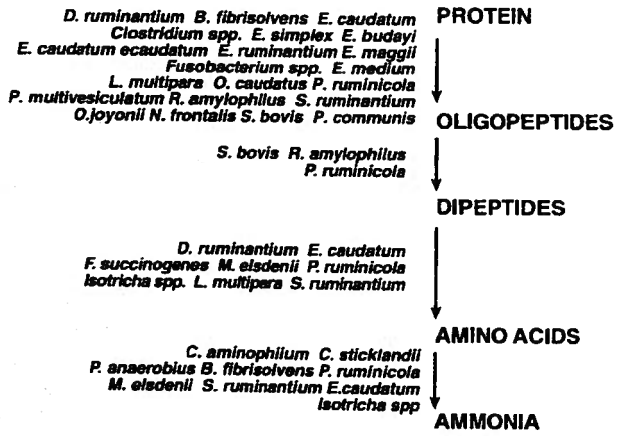

Figure 7.4 A summary of the microorganisms carrying out the various steps of the catabolic sequence from protein to ammonia in the rumen.

compounds, but their toxicity prohibits their use *in vivo* (Broderick and Balthrop, 1979).

The overall pattern of the microbial ecology of the conversion of protein to ammonia in the rumen can be viewed as an egg timer, with a constriction in the area of oligopeptide breakdown, where very few species are responsible for the catabolic activity and, at the present time, *P. ruminicola* appears most significant (Figure 7.4).

7.2.4 Breakdown of urea

Urea is broken down extremely rapidly in the rumen, releasing ammonia. This activity, combined with rumen microbial protein synthesis from ammonia, enables ruminants to utilize urea entering the rumen either with the feed (Virtanen, 1966; Roffler and Satter, 1975), or in endogenous salivary secretion, or by diffusion across the rumen wall (Kennedy and Milligan, 1980). The enzyme mechanism is a simple hydrolysis by urease, which can be inhibited *in vitro* by acetohydroxamic acid (Jones, 1968; Brent *et al.*, 1971; Cook, 1976; Makkar *et al.*, 1981). The rumen enzyme is probably similar to jackbean urease in its Ni content, judging by the stimulation of rumen urease activity by dietary Ni (Spears *et al.*, 1977; Spears and Hatfield, 1978). Urease is associated with the particulate microbial fraction of rumen fluid, and is predominantly of bacterial origin (Gibbons and McCarthy, 1957; Jones *et al.*, 1964; Mahadevan *et al.*, 1976). No urease activity was found in either starved, antibiotic-treated ciliate protozoa (Onodera *et al.*, 1977) or in *Piromyces* or *Neocallimastix* fungal isolates (Sakurada *et al.*, 1994). Urea is not hydrolysed in the absence of the microbial population, when its concentration in the rumen is the same as that in blood (Cheng and Wallace, 1979).

Precisely which bacterial species are most important in the hydrolysis of urea *in vivo* is not known, but the issue has provoked interesting speculation and discussion, which can be read in more detail elsewhere (Jones, 1967; John *et al.*, 1974; Wozny *et al.*, 1977; Hobson and Wallace, 1982). Some studies found strictly anaerobic bacteria which hydrolysed urea to be elusive (Jones *et al.*, 1964; Cook, 1976), but isolates from the genera *Lactobacillus*, *Peptostreptococcus*, *Propionibacterium*, *Bacteroides*, *Ruminococcus*, *Butyrivibrio*, *Treponema*, *Selenomonas*, *Bifidobacterium* and *Succinivibrio* have been obtained (Gibbons and Doetsch, 1959; Slyter *et al.*, 1968; John *et al.*, 1974; Wozny *et al.*, 1977). Ureolytic, facultatively anaerobic bacteria are more readily isolated from rumen contents. These have generally been present in smaller populations than the strict anaerobes, and include species of *Streptococcus*, *Staphylococcus*, *Micrococcus*, *Propionibacterium* and *Corynebacterium* (Cook, 1976; Cheng *et al.*, 1979; Wallace *et al.*, 1979; Cheng and Costerton, 1980). Their similarity to

the ureolytic population of the rumen wall prompted suggestions that these latter bacteria might be the more significant urease producers *in vivo* (Cheng *et al.*, 1979; Cheng and Costerton, 1980). Certainly, when the epithelial cells to which they are attached slough into rumen contents, wall bacteria confer a urease activity on the fluid sufficient to account for that found in normal rumen contents (Wallace *et al.*, 1979). The ecological argument is basically between a numerous population of strict anaerobes, mostly with a low urease activity, and a much smaller population of atypical, facultative anaerobes with characteristically high specific activity. There are weighty arguments in favour of each, and indeed the two populations may be equally important (Hobson and Wallace, 1982). One might speculate that, due to location, those organisms residing on the rumen wall may be most important in the hydrolysis of urea transferred across the rumen wall, while the organisms in the fluid may be important in the hydrolysis of dietary and salivary urea.

The urease activity present in rumen contents has been partially purified, and appears to be associated with a single polypeptide, of smaller molecular weight than jackbean urease (Mahadevan *et al.*, 1976). The urease of *Selenomonas ruminantium* strain D, which has been studied in some detail (John *et al.*, 1974; Wozny *et al.*, 1977; Smith and Bryant, 1979), differs from the enzyme purified from rumen contents in its specific activity, which is 20- to 30-fold higher than that of rumen urease, and its molecular weight, which is threefold greater (Hausinger, 1986). This should not be taken as evidence dismissing the other anaerobes – this strain was fairly atypical in any case in several of its properties – until similar work is done with other facultatively and strictly anaerobic urease producers.

Urease is one of the most variable enzyme activities in rumen contents. The effects of dietary Ni have been mentioned, but many other factors influence its activity. Ammonia may repress activity (John *et al.*, 1974; Cook, 1976; Wozny *et al.*, 1977; Cheng and Wallace, 1979), and urea is an inducer (Czerkawski and Breckenridge, 1982), but other regulatory factors are ill-understood, except in *S. ruminantium* (John *et al.*, 1974; Smith and Bryant, 1979). It is of interest to understand urease regulation, because the principal disadvantage of urea as a source of NPN is that it is broken down too rapidly, resulting in ammonia overflow and inefficient nitrogen retention.

7.2.5 Nucleic acids

Nucleic acids, despite comprising 5.2–9.5% of the total nitrogen in grasses and hay (Smith and McAllan, 1970; Coelho da Silva *et al.*, 1972), have received much less attention than proteins with regard to their breakdown by rumen microorganisms. DNA and RNA are rapidly hydrolysed in the rumen, whether added as pure compounds or as plant material (Smith and McAllan, 1970; McAllan and Smith, 1973*a*). Transient products formed are

a mixture of nucleotides, nucleosides and bases (McAllan and Smith, 1973*a*). McAllan and Smith (1973*b*) investigated the breakdown of nucleic acids in rumen fluid *in vitro*, and found that purine nucleotides formed hypoxanthine and xanthine, while pyrimidine nucleotides formed uracil and thymine. Cytosine was deaminated to uracil. Although these products were formed *in vitro*, no such accumulation was seen *in vivo*.

The microbial ecology of nucleic acid metabolism is poorly understood. For once, more is known about protozoal than bacterial metabolism, from the work of Coleman and his colleagues, but even then our knowledge is patchy. It is not known, for example, if protozoa are more important than the bacterial flora in this activity. Coleman (1968, 1980) showed that *Entodinium caudatum* assimilated bacterial nucleic acid components intact, and took up nucleotides from the medium. Several species, including *Entodinium caudatum* (Coleman, 1968), *Entodinium ecaudatum caudatum* (Coleman and Laurie, 1974), *Polyplastron multivesiculatum* (Coleman and Laurie, 1977) and *Eudiplodinium maggii* (Coleman and Sandford, 1979*b*), convert adenine and guanine into hypoxanthine and xanthine, metabolize pyrimidines, and incorporate exogenous bases. The principal function of these protozoal activities is thought to be the utilization of bacterial nucleic acids (Coleman, 1980).

Presumably the bacteria also degrade and incorporate nucleic acids, but derived mainly from the ruminant's food. Many strains of rumen bacteria produce extracellular nuclease activities that could be involved in DNA digestion (Flint and Thomson, 1990). These include strains of *Prevotella ruminicola*, *Fibrobacter succinogenes*, *Selenomonas ruminantium*, and *Lachnospira multipara*. Some ruminal bacteria can use adenine or guanine as a nitrogen source (Pittman and Bryant, 1964; John *et al.*, 1974), but this ability is probably linked to the deamination of these bases and use of the released ammonia. RNA-fermenting strains of *S. ruminantium* used ribonucleosides as sole sources of nitrogen and energy (Cotta, 1990). The purine nucleosides, adenosine and guanosine, supported much higher growth than pyrimidine nucleosides. Because bases alone did not support growth, it appeared that only the ribose moiety of the nucleosides was serving as an energy source. In these studies, DNA was not fermented, but deoxyribose-fermenting strains of *S. ruminantium* have been isolated (Rasmussen, 1993).

7.2.6 Other nitrogenous compounds in the diet

The other naturally occurring nitrogen-containing compounds in the diet that have received some attention are nitrate, ethanolamine and choline, and the possibilities that N_2 can be utilized from ingested air and diffuse from the blood to the rumen (Pun and Satter, 1975), and that novel non-protein nitrogen sources can be developed, have also been investigated.

Nitrate is quite abundant in some plant materials, and conceivably could

not only provide nitrogen for microbial protein synthesis but also provide a terminal electron acceptor for anaerobic respiration, thus enhancing energy production. Evidence for either of these possibilities is scant, however. Some strains of *Selenomonas ruminantium* use nitrate as a nitrogen source (John *et al.*, 1974), and presumably other rumen bacteria do so too. Nitrate metabolism *in vivo* can cause nitrite poisoning in the host animal if nitrite is not reduced to ammonium quickly enough (Lewis, 1951; Holtenius, 1957). Nitrate reduction was stimulated *in vitro* by the addition of H_2 or glucose as electron donors, and nitrite accumulated (Jones, 1972). Only formate was a good enough electron acceptor to prevent nitrite accumulation. Denitrification was considered to be unlikely in the rumen (Jones, 1972). Nothing is known of the organisms that are principally responsible for the different steps of nitrate reduction *in vivo*.

Choline is essential for the growth of the protozoon *Entodinium caudatum*, and is rapidly incorporated into phospholipid (Broad and Dawson, 1976). Ethanolamine is less rapidly used and cannot replace choline (Bygrave and Dawson, 1976). Indeed, choline incorporation is a good index of protozoal activity in the mixed rumen population (Newbold, personal communication). However, its main fate in the mixed population is to be converted into trimethylamine, which in turn is converted into methane (Itabashi and Kandatsu, 1978; Neill *et al.*, 1978) by the methanogen *Methanosarcina barkeri* (Patterson and Hespell, 1979).

The rate of N_2 fixation in the rumen is insignificant compared to the total nitrogen entering the rumen (Moisio *et al.*, 1969; Hobson *et al.*, 1973; Jones and Thomas, 1974). Even with daily inoculation of N_2-fixing *Bacillus macerans* into sheep on a 10% molasses diet, N_2 fixation in the rumen amounted to only $0.75\,g\,day^{-1}$ (Jones and Thomas, 1974).

Cheap, synthetic nitrogen-containing compounds that can yield ammonia by rumen microbial hydrolysis could be an attractive alternative to urea, particularly if they released ammonia at a rate balanced with the rate of energy production, unlike urea, which is broken down too quickly. The microbial population would probably have to adapt to these new substrates (Nikolic *et al.*, 1980), but compounds like biuret, creatine, ammoniated molasses and glycosyl ureas could be useful (Schwartz, 1967; Chalupa, 1972; Merry *et al.*, 1982). Little is known of the microbiology of their breakdown.

7.3 Anabolic nitrogen metabolism

7.3.1 Ammonia assimilation

Mixed rumen microorganisms. Ammonia is the most important source of nitrogen for protein synthesis in the rumen. ^{15}N-Labelling studies indicate

that between 42% and 100% of rumen microbial nitrogen is derived from ammonia (Pilgrim *et al.*, 1970; Mathison and Milligan, 1971; Al-Rabbat *et al.*, 1971*a*,*b*; Nolan *et al.*, 1976). The ammonia concentration in the rumen fluctuates markedly, from less than 1 mM observed in some animals on extremely low protein roughages to perhaps 40 mM, transiently after feeding, in animals receiving rapidly degraded protein or urea. There are several different enzymatic mechanisms for ammonia uptake into amino acids, each with a different affinity for its substrate. The most important mechanisms, and perhaps organisms as well, therefore probably vary as the ruminal ammonia concentration changes. Because it is the central pathway for protein synthesis, the mechanism of ammonia uptake is of great interest to microbiologists and nutritionists dealing with ruminants. Hespell (1984) dealt comprehensively with the microbiology and biochemistry of ammonia assimilation by rumen bacteria.

The first step in ammonia uptake into a cell is its transport across the cell membrane. Other than calculations suggesting that ammonia is accumulated within microbial cells (Russell and Strobel, 1987), nothing is known about this mechanism in rumen microorganisms. It is improbable that the rate of ammonia transport limits the rate of ammonia assimilation into amino acids; the synthesis of carbon skeletons is likely to be more problematic. Different enzyme systems may be used at different prevailing ammonia concentrations and depending on the degree of intracellular accumulation.

The highest-affinity enzyme system for ammonia assimilation is the glutamine synthetase–glutamate synthase (GS-GOGAT) couple (Brown *et al.*, 1974). Ammonia is first incorporated into the amide group of glutamine, using glutamate as substrate, and ATP is hydrolysed. The amide NH_2 is then transferred to α-oxoglutarate to form two molecules of glutamate. GOGAT has been demonstrated to be present in rumen microorganisms under conditions of low ammonia (Erfle *et al.*, 1977), but it is not significant at higher ammonia concentrations (Wallace, 1979; Lenartova *et al.*, 1985). The low K_m (ammonia) of GS (1.8 mM) (Woolfolk *et al.*, 1966) is consistent with an effective scavenging role at low rumen ammonia concentrations. Because ATP is required, however, this system might be expected to be a handicap to organisms at higher ammonia concentrations where ATP-independent enzymes can function. For this reason, the GS-GOGAT system is only expressed by *Klebsiella aerogenes*, for example, under conditions of ammonia limitation (Brown *et al.*, 1974). When fermentation *in vitro* was artificially ammonia-limited, GS activity increased 10-fold (Erfle *et al.*, 1977). Rumen ammonia concentrations *in vivo* would seldom be so low as to require the high affinity of the GS-GOGAT mechanism for effective assimilation.

Lower-affinity, higher K_m, systems present in rumen microorganisms include NADP–glutamate dehydrogenase (NADP-GDH; K_m for ammonia,

1.8–3.1 mM) (Wallace, 1979), NAD-GDH (20–33 mM) (Erfle *et al.*, 1977; Wallace, 1979) and alanine dehydrogenase (70 mM) (Wallace, 1979). Other possibilities that have been investigated have been asparagine synthetase replacing GS in a coupled system analogous to GS-GOGAT (Erfle *et al.*, 1977), NADP–alanine dehydrogenase and aspartate dehydrogenase (Wallace, 1979) and carbamyl phosphokinase (Chalupa *et al.*, 1970), but these activities were negligible or of very high K_m (ammonia).

NAD-linked GDH is the highest-activity ammonia-assimilating enzyme present, not only in rumen contents (Hoshino *et al.*, 1966; Palmquist and Baldwin, 1966; Chalupa *et al.*, 1970; Wallace, 1979; Bhatia *et al.*, 1980; Lenartova *et al.*, 1985) but also in rumen mucosa (Hoshino *et al.*, 1966; Chalupa *et al.*, 1970) and in bacteria attached to the rumen wall (Lenartova *et al.*, 1985). Sometimes this activity is much higher than that of the NADP-linked enzyme (Chalupa *et al.*, 1970; Wallace, 1979), but activity varies with diet, and the two activities can be quite similar (Erfle *et al.*, 1977; Lenartova *et al.*, 1985). Under the latter circumstances, the higher K_m (ammonia) of NAD-GDH would restrict its activity to one primarily of glutamate catabolism.

Various aminotransferase (transaminase) activities are present in rumen contents, which transfer the trapped NH_2 from the primary amino acid throughout the amino acid pool. The most commonly found are glutamate–pyruvate and glutamate–oxaloacetate aminotransferases (Chalupa *et al.*, 1970; Bhatia *et al.*, 1979; Wallace, 1979; Lenartova *et al.*, 1985), but many others exist to disperse the bound ammonia (Bhatia *et al.*, 1979).

A question then arises as to which of these mechanisms actually provides the main route of ammonia assimilation. From the enzymatic observations, glutamate would be expected to be the first amino acid into which ammonia would be assimilated, and this coincides with glutamate usually being the most abundant amino acid in the free amino acid pool (Wright and Hungate, 1967; Shimbayashi *et al.*, 1975; Erfle *et al.*, 1977; Wallace, 1979; Blake *et al.*, 1983). However, alanine was surprisingly prominent in these pools and often exceeded glutamate, particularly under conditions of high ammonia concentration. Clearly, this need not mean that alanine is the primary product of ammonia assimilation. Imbalances in rates of formation by transaminases and rates of utilization in protein synthesis could easily result in alanine accumulation. However, the earlier indications of Shimbayashi *et al.* (1975) that alanine was a primary product were confirmed by Blake *et al.* (1983). [^{15}N]Ammonium chloride enriched alanine more than glutamate or other amino acids in the microbial pool after only 2 min (Blake *et al.*, 1983). The role of alanine and alanine dehydrogenase in ammonia assimilation is still in need of clarification. Past experience would suggest that the correct electron donor has not been found (Hespell, 1984). Furthermore, given the high K_m (ammonia) and low activity of alanine dehydrogenase that has so far been measured, the pos-

sible energy-linked accumulation of ammonia within cells, as occurs with *E. coli* (Stevenson and Silver, 1977) and *Clostridium pasteurianum* (Kleiner and Fitzke, 1979), then becomes critical to the efficient assimilation of ammonia into alanine.

The different enzymatic mechanisms for ammonia uptake probably reflect different niches that the microorganisms occupy. For example, the maximum rate of degradation of barley in the rumen occurs at an ammonia concentration of 9 mM or above (Mehrez *et al.*, 1977; Wallace, 1979; Odle and Schaefer, 1987), higher than that required for corn (Slyter *et al.*, 1979; Nikolic and Filipovic, 1981; Odle and Schaefer, 1987), and much higher than the K_s (ammonia) of predominant rumen bacteria (less than 50 μM) (Schaefer *et al.*, 1980). It has been suggested that this apparent excess of ammonia in total rumen contents may be necessary for sufficient penetration of ammonia to the site of digestion of a feed component. Ammonia could easily be limiting within that microenvironment, depending on the nature of the diet. The microenvironment probably varies enormously from one plant material to another, explaining the different effects of gross ammonia concentration on the rate at which different feeds are fermented. Furthermore, it would also explain why rumen bacteria retain the ability to form the GS-GOGAT system when ammonia becomes limiting (Erfle *et al.* 1977; Hespell, 1984).

Individual species. Ciliate protozoa are known to synthesize some of their amino acids *de novo* (reviews: Coleman, 1980; Williams, 1986; Williams and Coleman, 1992), but to our knowledge the extent to which ammonia is necessary for protein synthesis, and the assimilation mechanism, have not been described. The fact that the anaerobic fungus, *Neocallimastix frontalis*, grew in a defined medium without preformed amino acids (Lowe *et al.*, 1985) suggested that these organisms may depend heavily on ammonia for protein synthesis *in vivo*. Again, information on the enzymatic mechanism of uptake is lacking.

Most species of rumen bacteria can use ammonia as their main source of nitrogen for growth (Bryant, 1974), and indeed appear to do so under conditions normally prevailing in the rumen (Nolan, 1975). Ammonia is in fact essential for the growth of many species (Allison, 1969, 1970; Bryant, 1974). The enzymology of ammonia uptake has been studied in some of these species.

Ammonia-limited growth of *Ruminobacter amylophilus* resulted in a repression of the main glutamate dehydrogenase activity, NADP-GDH, and a stimulation of GS (Jenkinson *et al.*, 1979). GOGAT activity was not detected (Jenkinson *et al.*, 1979), implying that the high-affinity GS-GOGAT couple could not function. Several mechanisms for ammonia assimilation are therefore possible. The first is that NADP-GDH, with its K_m (ammonia) of 1.0–1.7 mM, is the principal ammonia uptake enzyme. In

that case, the very low ammonia saturation constant (6–13 μM) (Schaefer *et al.*, 1980) for the whole organism would have to be explained by an active accumulation of ammonia intracellularly. Alternatively, GS may indeed be the first uptake enzyme, with the secondary aminotransferase activity being different from GOGAT. As in the mixed population, alanine was prominent in the intracellular free amino acid pools, yet alanine dehydrogenase was low (Jenkinson *et al.*, 1979).

With *Selenomonas ruminantium*, GS activity was again stimulated in ammonia-limited cultures (Smith *et al.*, 1980, 1981; Hespell, 1984), particularly at high growth rate (Hespell, 1984). Unlike for *Ruminobacter amylophilus*, a low GOGAT activity was found in *Selenomonas ruminantium* (Smith *et al.*, 1981), so the GS-GOGAT couple would be expected to function at low ammonia concentrations. Surprisingly, GOGAT was not induced by ammonia limitation (Smith *et al.*, 1981). NADP-GDH activity was higher in glucose-limited (ammonia-sufficient) cultures, suggesting that it was the route used under these conditions (Smith *et al.*, 1980; Hespell, 1984). Hespell (1984) calculated that 50% of the difference in growth yields obtained in glucose- and ammonia-limited chemostats could be accounted for by a switch from GDH to the ATP-consuming GS-GOGAT route of ammonia uptake.

The NADP-GDH of *Ruminococcus flavefaciens* has been purified, its N-terminal sequence determined, and some of its kinetic properties analysed (Duncan *et al.*, 1992). The enzyme has a K_m for ammonia of 19 mM, and yet it was induced in ammonia-limited cultures where the ammonia concentration was 0.1 mM or less (Pettipher and Latham, 1979; Duncan *et al.*, 1992). Active accumulation of ammonia might be expected to account for this anomaly, but no accumulation of [^{14}C]methylamine could be demonstrated (Duncan *et al.*, 1992). The N-terminal sequence suggested that the protein may be modified post-translationally and that it differs significantly from NADP-GDHs of *E. coli*, *Salmonella typhimurium* and *Clostridium symbiosum* (Duncan *et al.*, 1992).

The gene for the GS of *Butyrivibrio fibrisolvens* is so far the only major nitrogen-metabolism gene of a rumen organism which has been cloned and sequenced (Goodman and Woods, 1993). This GS gene was only the second reported example of a type III gene, previously found only in *Bacteroides fragilis*, a major member of the human colonic microflora.

Other species have been less well studied. *Streptococcus bovis* possesses GS (Griffith and Carlsson, 1974) and NADP-GDH (Burchall *et al.*, 1964; Griffith and Carlsson, 1974), but not GOGAT (Griffith and Carlsson, 1974). NADP-GDH activity was much higher in ammonia-limited cells, and it was concluded that this was the only pathway of ammonia assimilation in *Streptococcus bovis* (Griffith and Carlsson, 1974). *Succinivibrio dextrinosolvens* has both NADP-GDH and GS activities (Patterson and Hespell, 1985).

In all of these pure cultures, the mechanism of ammonia uptake has been inferred from the enzymes present, and by the way that they are regulated. The work of Smith *et al.* (1980), Hespell (1984) and others has shown that the conditions for measurement of the enzymes really must be investigated in detail before such a conclusion can be drawn. In fact, the only sure way of establishing the true mechanism is to use a ^{13}N or ^{15}N technique using labelled ammonia.

7.3.2 Amino acid biosynthesis

There is evidence for *de novo* synthesis of amino acids in protozoa. ^{14}C-Labelled monosaccharides were incorporated into the protein of holotrichs (Williams and Harfoot, 1976; Williams, 1979), as was ^{14}C-labelled sodium carbonate, which was incorporated into alanine, histidine, threonine, glutamate and aspartate (Harmeyer, 1965). Ciliates form lysine from diaminopimelic acid which is present in the cell wall peptidoglycan of the bacteria which they ingest (Onodera and Kandatsu, 1974; Onodera *et al.*, 1974; Masson and Ling, 1986), and presumably many of the other amino acids are incorporated direct, after the digestion of bacteria, or are formed from pre-existing carbon skeletons derived from the bacteria. For example, the rumen ciliates can produce tryptophan from indolepyruvate, which is formed from indoleacetate by the reductive carboxylation of the rumen bacteria (Okuuchi *et al.*, 1993).

Rumen fungi are able to grow in media lacking preformed amino acids and therefore must be able to synthesize the needed amino acids, but information on amino acid biosynthesis is lacking (Lowe *et al.*, 1985). Amino acids, particularly aromatic amino acids, were stimulatory to growth (Orpin and Greenwood, 1986), suggesting that proteolytic activity might enhance fungal competitiveness in the rumen ecosystem by providing stimulatory peptides and amino acids. However, little is known about the amino acid metabolism of rumen fungi, in contrast to the bacteria, which have been studied for a much longer time.

Information is most plentiful for amino acid biosynthesis by rumen bacteria. The biosynthetic pathways for the production of amino acids by bacteria and fungi have been reviewed by Umbarger (1978), and the reader is referred to this work for a more detailed discussion of the synthesis of individual amino acids. In brief, amino acids can be divided into groups or families based on the source of carbon used for their synthesis. These are: the glutamate family – glutamate, glutamine, proline, arginine; the serine family – serine, glycine, cysteine; the aspartate family – aspartate, asparagine, lysine, methionine, threonine, isoleucine; the pyruvate family – alanine, isoleucine, leucine, valine; the aromatic family – phenylalanine,

tyrosine, tryptophan; and histidine. While absolute proof of the synthesis of all amino acids by the same pathways in rumen bacteria is lacking, evidence for a number of these pathways exists and radioactive tracer experiments on amino acid synthesis by mixed rumen bacteria yielded amino acids with labelling patterns consistent with the pathways described by Umbarger (Sauer *et al.*, 1975). As illustrated in the section on ammonia assimilation by rumen microorganisms, glutamic acid occupies a central role in the nitrogen metabolism of organisms, and thus the generation of α-oxoglutarate is of great importance to nitrogen metabolism in rumen bacteria. Since these bacteria are anaerobes, and lack a functional tricarboxylic acid (TCA) cycle, α-oxoglutarate is not produced as a normal intermediate of energy metabolism as in aerobic organisms. Synthesis of α-oxoglutarate by rumen bacteria has been examined using both mixed and pure cultures of bacteria. Milligan (1970) showed that rumen contents incubated with $NaH^{14}CO_3$ produced glutamate labelled in the C-1, C-2 and C-5 positions. This labelling pattern suggested that α-oxoglutarate was synthesized by both forward and reverse TCA cycle activity. That is, α-oxoglutarate is formed by reductive carboxylation of succinic acid for the reverse TCA cycle, as against condensation of oxaloacetic acid and acetyl-CoA to form citrate and subsequent forward TCA activities. The specific activity of labelled glutamate carbons indicated that the reverse TCA cycle route of α-oxoglutarate synthesis was the predominant pathway employed by rumen microorganisms. Later experiments by Sauer *et al.* (1975), using a mixed rumen microbial population maintained in continuous culture, confirmed the presence of both modes of α-oxoglutarate synthesis. In their experiments, however, forward TCA cycle function was concluded to be the major pathway of α-oxoglutarate generation. Representatives of individual species of rumen bacteria can be cited for both mechanisms of α-oxoglutarate synthesis.

Megasphaera elsdenii produces α-oxoglutarate by the forward TCA method of synthesis. This was first suggested by specific labelling of glutamate carbons when this organism was provided with [1-^{14}C]lactate as the growth substrate (Somerville and Peel, 1967). Somerville (1968) demonstrated that *M. elsdenii* produces the required enzymatic machinery for this synthesis. Allison and Robinson (1970) showed that *Prevotella ruminicola* forms α-oxoglutarate by reductive carboxylation of succinate and described some characteristics of the α-oxoglutarate synthase reaction. Subsequently, strains of *Selenomonas ruminantium*, *Veillonella alcalescens* and other gastrointestinal tract *Bacteroides* spp. were shown to synthesize α-oxoglutarate by reductive carboxylation of succinate (Allison *et al.*, 1979). Conversion of [^{14}C]succinate into [^{14}C]glutamate could not be demonstrated for *Ruminococcus flavefaciens*, *Methanobrevibacter ruminantium*, *Streptococcus bovis*, *Butyrivibrio fibrisolvens* and *Succinivibrio dextrinosolvens*, but these strains failed to take up exogenous [^{14}C]succinate, and

this evidence does not preclude existence of this pathway for α-oxoglutarate synthesis.

Direct demonstration of the production of the other important carbon skeletons for amino acid biosynthesis is variable. Pyruvate is generated in the energy metabolism of the majority of rumen bacteria and can also be produced by the reductive carboxylation of acetate (Allison, 1969; Prins, 1977). Serine is produced from phosphoglyceric acid (a glycolytic intermediate) by conversion of this compound into phosphohydroxypyruvate and then phosphoserine and serine (Somerville, 1968; Sauer *et al.*, 1975). Aspartate is the transamination product of oxaloacetic acid which is produced in the energy metabolism of most succinic acid-producing rumen bacteria (Joyner and Baldwin, 1966; Prins, 1977). Oxaloacetic acid is also generated in both of the pathways of α-oxoglutarate synthesis discussed previously. Evidence for the biosynthetic pathways resulting in the formation of the aromatic amino acids and histidine is more indirect. The results of the radioactive tracer studies of Sauer *et al.* (1975) are consistent with the synthesis of histidine from phosphoribosyl pyrophosphate and aromatic amino acids via the shikimic acid pathway, although the results suggest that this may not be the major route of aromatic amino acid synthesis used by rumen bacteria.

While a number of rumen bacteria can form all the carbon skeletons needed for amino acid synthesis, many organisms have the ability to utilize products formed by other organisms as intermediates in the synthesis of amino acids. In fact, in many cases bacteria have an absolute nutritional requirement for such intermediates. Probably the most widely recognized example of this phenomenon is the branched-chain VFA requirement of the predominant cellulolytic rumen bacteria. *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* and *R. albus* require one or more of the branched-chain fatty acids isobutyric acid, 2-methylbutyric acid and isovaleric acid for the synthesis of valine, isoleucine and leucine respectively (Bryant and Robinson, 1962; Allison and Bryant, 1963; Dehority *et al.*, 1967). Conversion of the branched-chain fatty acid into the appropriate amino acid is the result of reductive carboxylation and transamination of the fatty acid. Other organic acids that can be converted into amino acids in this manner include phenylacetate and indoleacetic acid, for the synthesis of phenylalanine and tryptophan respectively (Allison, 1965; Allison and Robinson, 1967). It is interesting that organisms such as *P. ruminicola* and *M. elsdenii*, which produce the branched-chain fatty acids, can also utilize these for the synthesis of amino acids (Allison and Peel, 1971; Allison, 1969). Allison *et al.* (1984) found that, when *P. ruminicola* was grown in media lacking isovaleric acid, carbon from glucose was used for the synthesis of leucine. However, when isovaleric acid was added to the medium, the conversion of [^{14}C]glucose into leucine was markedly inhibited. They also found that synthesis of phenylalanine or isoleucine from glucose was re-

duced when phenylacetate or 2-methylbutyrate were provided in the medium. The authors concluded that this organism has the ability or regulate these pathways of amino acid biosynthesis, and will use preformed intermediates for synthesis of these amino acids in preference to *de novo* synthesis. Furthermore, since these intermediates are generally present in rumen fluid, their reductive carboxylation is likely to be the predominant pathway for the synthesis of the related amino acids in the rumen.

Most pure-culture work on bacterial peptide metabolism has been done with *P. ruminicola*. Peptides did not support the growth of this organism in the absence of an energy source, and even when glucose was present the energy derived from the subsequent deamination was relatively small (Russell, 1983). Peptides such as the octapeptides oxytocin and vasopressin, or enzyme-hydrolysed casein, were, however, able to replace ammonia as the main nitrogen source for growth, whereas small peptides of less than four residues, free amino acids or some other low molecular weight compounds could not (Pittman and Bryant, 1964). The oligopeptides are hydrolysed on entering the cells and simply provide intracellular amino acids for growth (Pittman *et al.*, 1967). Amino acid transport systems do occur in *P. ruminicola*, but they appear to be inhibited by a substance, possibly acetate, in the medium (Stevenson, 1979). It is therefore likely that, given the presence of high concentrations of VFA in the rumen, *P. ruminicola* will utilize mainly peptides and ammonia *in vivo*. It is not known if the same applies to other species of rumen bacteria that grow better in the presence of enzyme-hydrolysed casein.

7.3.3 The requirement for rumen-degradable protein

Are the rumen fermentation and rumen microbial growth ever limited by the availability of preformed amino acids, as opposed to ammonia? If so, which amino acids are stimulatory and when does this need arise? And in what form should the amino acids be – free or peptide-bound? These questions are directly relevant to practical animal nutrition.

As mentioned at the beginning of the chapter, the mixed rumen microbial population has no absolute amino acid requirement (Virtanen, 1966), but there is ample evidence that amino acids and especially peptides are stimulatory in terms of both growth rate and growth yield for rumen microorganisms growing on rapidly degraded energy sources (Figure 7.5; Table 7.1; Russell *et al.*, 1983; Russell, 1983; Chen *et al.*, 1987*a*; Argyle and Baldwin, 1989; Cruz Soto *et al.*, 1994). The energy substrate in these studies comprised soluble sugars, however, and the same may not be true for energy substrates that are degraded more slowly. Cruz Soto *et al.* (1994) found that the infusion of amino acids or peptides directly into the rumen had no influence on fermentation rate or microbial protein synthesis. The reason suggested for this observation was that the cellulolytic bacteria were

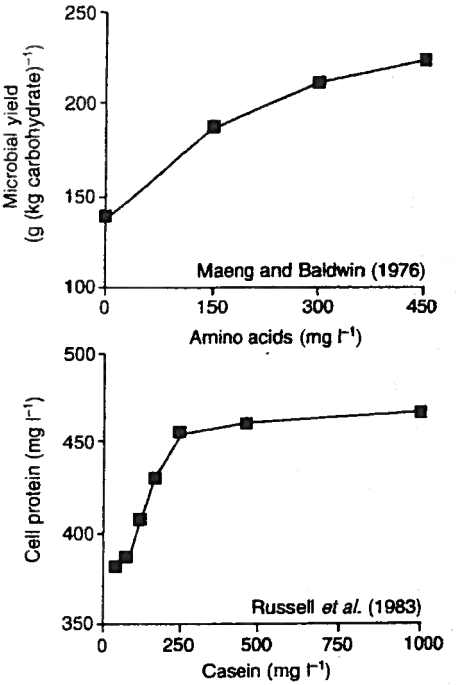

Figure 7.5 Influence of amino acids on growth of mixed rumen bacteria.

Table 7.1 Comparison of effects of peptides and amino acids on growth yield of mixed rumen microorganisms

Amino acid/Trypticase concentration (mg l^{-1})	Bacterial yield (μg RNA/culture)	
	Amino acids	Trypticase
0	110	110
1	170	171
10	197	220[a]
1000	263	267

[a] Significent difference ($P < 0.05$).
Data from Argyle and Baldwin (1989).

stimulated by preformed amino acids when growing on cellobiose, but not when growing on cellulose, where the rate of digestion of cellulose was the most important factor limiting growth rate. A subsequent study (Chikunya *et al.*, 1996) demonstrated that rumen fermentation was stimulated by sol-

uble protein when fibre was rapidly fermented, but not on more slowly degraded fibre. The Cornell model (Russell *et al.*, 1992) distinguishes between microorganisms fermenting structural and those hydrolysing non-structural carbohydrates, and assumes that digestion of the former is carried out by bacteria that require only ammonia as a source of nitrogen while the species that break down non-structural carbohydrates will benefit from preformed amino acids. Thus there seems little doubt that amino acids, compared with ammonia, will benefit rumen fermentation under some circumstances, but the precise relation between energy source, microbial growth rates and the benefits of amino acids/peptides probably requires further refinement. It is also well known to practical rumen microbiologists that peptides rather than amino acids support better growth of most cultures, and the evidence for this was described earlier. However, the only evidence that peptides are superior to free amino acids in the mixed population was obtained under only one of the combinations of conditions investigated by Argyle and Baldwin (1989) (Table 7.1). Otherwise, free amino acids provided as much benefit.

7.4 Conclusion

A great deal of research has been devoted to the biochemical mechanisms and microbial ecology which are involved in nitrogen metabolism by rumen microorganisms. A broad understanding has emerged, with inevitable questions remaining and gaps and contradictions evident. However, molecular studies to back up these observations have been slow in appearing, despite the obvious nutritional importance of the topic. It is to be hoped that this situation will change soon, such that the genetic mechanisms which control this vital area of rumen metabolism can be elucidated.

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